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Effect of Insulin Sensitizer Therapy on Amino Acids and Their Metabolites☆

Brian A. Irving[a,*], Rickey E. Carter[b], Mattias Soop[a], Audrey Weymiller[c], Husnain Syed[d], Helen Karakelides[a], Sumit Bhagra[a], Kevin R. Short[a], Laura Tatpati[e], Rocco Barazzoni[a], K. Sreekumaran Nair[a,**]

[a] Division of Endocrinology, Endocrinology Research Unit, Mayo Clinic College of Medicine, Rochester, MN
[b] Department of Health Sciences Research, Mayo Clinic College of Medicine, Rochester, MN
[c] Department of Nursing, Mayo Clinic College of Medicine, Rochester, MN
[d] Department of Family Medicine, Mayo Clinic College of Medicine, Rochester, MN
[e] Division of Reproductive Endocrinology, Mayo Clinic College of Medicine, Rochester, MN

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ABSTRACT

Aims. Prior studies have reported that elevated concentrations of several plasma amino acids (AA), particularly branched chain (BCAA) and aromatic AA predict the onset of type 2 diabetes. We sought to test the hypothesis that circulating BCAA, aromatic AA and related AA metabolites decline in response to the use of insulin sensitizing agents in overweight/obese adults with impaired fasting glucose or untreated diabetes.

Methods. We performed a secondary analysis of a randomized, double-blind, placebo, controlled study conducted in twenty five overweight/obese (BMI ~30 kg/m^2) adults with impaired fasting glucose or untreated diabetes. Participants were randomized to three months of pioglitazone (45 mg per day) plus metformin (1000 mg twice per day, N = 12 participants) or placebo (N = 13). We measured insulin sensitivity by the euglycemic-hyperinsulinemic clamp and fasting concentrations of AA and AA metabolites using ultra-pressure liquid chromatography tandem mass spectrometry before and after the three-month intervention.

Results. Insulin sensitizer therapy that significantly enhanced insulin sensitivity reduced 9 out of 33 AA and AA metabolites measured compared to placebo treatment. Moreover, insulin sensitizer therapy significantly reduced three functionally clustered AA and metabolite pairs: i) phenylalanine/tyrosine, ii) citrulline/arginine, and iii) lysine/α-aminoadipic acid.

Conclusions. Reductions in plasma concentrations of several AA and AA metabolites in response to three months of insulin sensitizer therapy support the concept that reduced insulin sensitivity alters AA and AA metabolites.

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* Correspondence to: B.A. Irving, Geisinger Obesity Institute, 100 N. Academy Ave, Geisinger Health System, Danville, PA 17868. Tel.: +1 271 5555x55234.

** Correspondence to: K.S. Nair, Endocrinology Division, 200 First Street SW, Joseph 5–193, Mayo Clinic, Rochester, MN 55905. Tel.: +1 507 255 2415; fax: +1 507 255 4828.

E-mail addresses: bairving@geisinger.edu (B.A. Irving), nair@mayo.edu (K.S. Nair).

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1. Introduction

In people with insulin resistance plasma concentrations of branched chain amino acids (BCAA; leucine, isoleucine, and valine), aromatic amino acids (AAA; phenylalanine and tyrosine), and amino acid (AA) metabolites are elevated [1–4]. Moreover, BCAA were recently reported to be elevated in metabolically well compared to metabolically unwell adults, independent of obesity [5,6]. Importantly, elevations in BCAA and AAA occur approximately a decade prior to the development of type 2 diabetes (T2D), which has led to the proposal that these AA are useful predictors for future T2D [7]. *In vitro*, supraphysiological doses of AA impair insulin signaling at several steps critical for glucose uptake [8] and glycogen synthesis [9]. *In vivo*, the infusion of AA, especially BCAA, reduces insulin sensitivity [10–12]. BCAA deprivation in rodents also improves insulin sensitivity in insulin resistant mice [13]. On the other hand, the rapid increase in insulin sensitivity and improvement in glucose homeostasis following weight loss surgery coincides with reductions in circulating BCAA, AAA, and several AA metabolites [14–16]. In addition, three months of metformin monotherapy has been reported to reduce AAA in patients with T2D [17]. Therefore, monitoring changes in BCAA, AAA, and AA metabolites may provide insight into the early responses to T2D therapies [18]. The close association between elevations AA, especially BCAA and AAA, and insulin resistance has led to the hypothesis that elevations in BCAA and AAA cause insulin resistance [1]. However, another equally plausible hypothesis is that reductions in the activity of branched chain ketoacid dehydrogenase (BCKD) and tyrosine aminotransferase (TAT) in states of insulin resistance lead to increased tissue and circulating BCAA and AAA concentrations, respectively [19].

We proposed that pharmacologically improving insulin sensitivity would result in reductions in plasma concentrations of BCAA, AAA, or other AA metabolites in insulin-resistant adults. Insulin is a potent antiproteolytic hormone that results in hypoaminoacidemia when systemically administered [20–22] due to a concomitant reduction in skeletal muscle protein breakdown [23] and increased AA transport into the muscle [24]. Previous studies have also shown that insulin deprivation in c-peptide negative adults with type 1 diabetes increases protein turnover (i.e., synthesis and breakdown) [25], AA oxidation [25], and transamination rates of leucine [26], and is accompanied by large increases in circulating AA concentrations, especially BCAA [26]. Moreover, protein degradation in multiple tissues is increased in people with T2D compared to non-diabetic controls under hyperglycemic conditions [27]. As stated previously, reductions in BCKD activity, TAT activity, and amino acid oxidation in states of insulin resistance lead to increased tissue and circulating BCAA and AAA concentrations [19]. Reductions in the degradation of BCAA also could lead to reductions in branched chain α-ketoacids concentrations and the synthesis of monomethyl branched-chain fatty acids with adipose tissue and perhaps muscle [28]. Monomethyl branched-chain fatty acids have recently been demonstrated to positively correlate with insulin sensitivity [28]. Together these data support the premise that a reduction in insulin action contributes to elevations in tissue and circulating BCAA, AAA, and AA metabolite concentrations. Since it has been reported that BCAA, especially leucine, can inhibit insulin action [1] there may be a type of feed-forward relationship between insulin resistance and the concentration of circulating AA.

The current study was designed to determine whether chronically enhancing insulin sensitivity reduces plasma BCAA, AAA, and AA metabolites in insulin resistant adults. Specifically, we investigated the impact of three months of dual insulin sensitizer therapy (45 mg pioglitazone per day plus 1000 mg metformin twice per day) in overweight/obese adults who had impaired fasting glucose or untreated diabetes.

2. Methods

The Mayo Clinic's Intuitional Review Board approved the study protocol in accordance to the principles of the Declaration of Helsinki. All participants provided written and informed consent prior to participation.

2.1. Study Design and Participants

We previously reported the overall study design for the parent study [29]. The current report primarily examines the effect of three months of insulin sensitizer therapy on plasma concentrations of BCAA, AAA, and AA metabolites in overweight/obese adults with fasting hyperglycemia, defined as either impaired fasting glucose or untreated diabetes [29]. Briefly, 25 drug naïve, Northern European American participants with fasting blood glucose concentrations of 108–180 mg/dL were randomized to receive either 45 mg of pioglitazone per day plus 1 g of metformin twice per day (n = 12) or placebo (n = 13) for 12 weeks. We chose metformin based on its proven effect on hepatic insulin sensitivity and pioglitazone based on its effect on peripheral insulin sensitivity. Current use of hypoglycemic medications excluded participants from the present study.

Insulin sensitivity was measured using a hyperinsulinemic-euglycemic clamp as previously described [29]. In brief, participants received a continuous infusion of insulin (1.5 mU/kg-FFM/min) for 8 hours. Participants also received a continuous infusion of AA (5.4% NephrAmine, B. Braun Medical Inc.) to prevent insulin-induced hypoaminoacidemia. The AA analyses in the current report were performed on fasting blood samples that were acquired before any infusions began. Arterialized-venous blood was used to measure plasma glucose concentrations every 10 min using an automated glucose analyzer (GM9, Analox Instruments, London, UK). The glucose infusion rate (GIR) (40% dextrose) was adjusted to maintain euglycemia [~5.0 mmol/L (90 mg/dL)] during the 8 h insulin infusion. Insulin sensitivity was defined as the steady-state glucose infusion rate (GIR, μmol/kg-FFM/min) achieved during the last 2 h of the 8 h hyperinsulinemic-euglycemic clamp [30]. Fasting glucose and insulin concentrations were used to calculate the Quantitative Insulin Sensitivity Check Index (QUICKI = 1/log[glucose] + log[insulin]) [31]. Fasting plasma AA and AA metabolite concentrations were measured using ultra-pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

in the Mayo Clinic Metabolomics Core as previously described [32]. In brief, we drew arterialized-venous blood samples into plasma EDTA tubes, processed, and stored them at −80°C until analysis. Storage of plasma/serum samples at −80°C for qualitative and quantitative metabolomics using mass spectrometry are routinely done, demonstrating little to no evidence of storage related decay including samples stored greater than 20 years [7,33]. The frozen samples were thawed on ice and spiked with C^{13}-labeled internal standards and processed for analysis as previously described [32]. Amino acids in particular are relatively stable for several years, with the exception of their keto-acids (e.g., KIC), which we have not included in the present analysis. Subsequently, we separated the samples using an Acquity UPLC system, followed by mass detection using a TSQ Ultra Quantum from Thermo Finnigan running in ESI positive mode as previously described [32]. We calculated the AA and AA metabolites concentrations using 10 point standard curves as previously described [32]. We handled the pre- and post-intervention samples similarly and analyzed them in the same run. The reproducibility of the UPLC/MS/MS method in the MCRMC is excellent (average CV ~9.5%) [32].

Additionally, we measured plasma AA and AA metabolites in samples collected during a second study in which an acute (7-hours) infusion of insulin (1.5 mU/kg FFM/min) with glucose replacement and without AA replacement compared to saline alone (n = 9 each, both groups 5 M/4 F) [34]. The original goal of that protocol was to assess the effect of insulin on muscle protein metabolism in young, healthy, normal weight men and women. We previously reported that the plasma concentrations of 16 AA (out of 17 measured) declined during the 7 hour insulin infusion [34]. For the current report, we expanded the panel of measured AA and AA metabolites. The rationale for including those analyses in the current report is to help determine whether changes in AA and AA metabolites following insulin sensitizer therapy were attributable to the improvement of insulin action or to the direct effect of the pharmacological agents.

2.2. *Metabolic Assessment*

All measurements for both studies were performed in the Mayo Clinic's Center for Translational Science Activities' Clinical Research Unit (CRU). Body composition was measured using dual-energy x-ray absorptiometry (Lunar DPX-L; Lunar Radiation, Madison, WI).

Table 1 – Amino acid and amino acid metabolite concentrations (μmol/L) at baseline stratified by treatment group. Data presented as median (*interquartile range*).

	Placebo	Pioglitizone plus Metformin	*Wilcoxon-Rank Sum* P-Value*
N	13	12	-
Leucine	141.4 (123.3, 189.4)	142.2 (122.4, 169.9)	0.683
Isoleucine	70.6 (59.3, 93.4)	66.4 (56.9, 87.2)	0.497
Valine	276.7 (254.4, 324.9)	260.2 (241.5, 334.1)	0.532
Phenylalanine	59.4 (57.4, 64.9)	58.0 (52.3, 63.9)	0.765
Tyrosine	74.3 (56.8, 88.1)	74.6 (67.0, 91.8)	0.369
Lysine	193.3 (170.8, 228.6)	207.1 (196.2, 238.1)	0.201
Arginine	75.6 (68.3, 94.1)	88.8 (75.0, 99.1)	0.462
Methionine	20.8 (16.8, 26.4)	22.0 (19.6, 26.2)	0.314
Glutamine	590.4 (268.5, 769.4)	616.7 (249.2, 740.8)	0.807
Threonine	112.6 (108.0, 151.1)	115. 8 (101.6, 151.9)	>0.999
Alanine	384.9 (319.5, 409.0)	359.7 (298.4, 393.9)	0.644
Aspartic acid	2.1 (1.8, 2.8)	2.1 (1.8, 2.4)	0.765
Glutamic acid	63.5 (40.1, 71.4)	59.5 (47.1, 68.1)	0.849
Serine	84.7 (76.4, 106.7)	78.5 (72.0, 94.9)	0.644
Glycine	153.9 (137.3, 191.6)	157.2 (134.7, 218.8)	0.892
Histidine	76.1 (72.7, 83.7)	69.3 (58.9, 77.8)	0.121
L-Methylhistidine	16.2 (10.9, 18.3)	8.1 (6.9, 10.9)	0.077
3-Methylhistidine	5.7 (5.1, 6.7)	4.7 (4.3, 6.1)	0.221
β-Alanine	2.7 (2.0, 3.2)	2.5 (2.1, 2.8)	0.683
α-Aminoadipic-acid	1.2 (0.7, 1.3)	1.0 (0.7, 1.3)	0.369
β-Aminoisobutyric-acid	0.9 (0.8, 1.0)	0.8 (0.6, 1.3)	0.573
α-Amino-N-butyric-acid	24.7 (19.9, 29.6)	26.4 (22.2, 31.0)	0.605
Asparagine	59.8 (52.5, 66.2)	63.3 (54.3, 66.3)	0.683
Citrulline	31.4 (28.6, 35.8)	32.2 (25.5, 34.9)	0.724
Ornithine	51.5 (37.9, 67.4)	53.2 (44.7, 60.3)	0.978
Taurine	46.0 (40.5, 49.6)	40.6 (35.7, 47.3)	0.242
Ethanolamine	6.0 (5.0, 7.2)	6.2 (5.1, 6.9)	0.978
Sarcosine	1.2 (1.1, 1.4)	1.1 (1.0, 1.4)	0.765
Proline	192.0 (152.8, 240.0)	199.7 (176.6, 221.3)	0.849
Hydroxyproline	7.3 (7.0, 9.4)	7.3 (6.2, 8.2)	0.463
Hydroxylysine 2	0.4 (0.3, 0.5)	0.3 (0.2, 0.4)	0.849
Cystine	76.1 (54.0, 82.5	90.8 (32.6, 106.6)	0.369
Tryptophan	57.6 (53.2, 59.5)	60.9 (50.0, 65.5)	0.605

* Wilcoxon Rank Sum, normal approximation.

Table 2 – Pre-to-post intervention change in amino acid and amino acid metabolite concentrations (μmol/L) in response to three months of insulin sensitizer therapy or placebo. Data presented as median (*interquartile range*). *Wilcoxon Rank Sum, normal approximation.

	Placebo	Pioglitizone plus Metformin	*Wilcoxon-Rank Sum* P-Value*
n	13	12	-
Leucine	13.3 (8.5, 22.8)	6.3 (−3.9, 16.8)	0.369
Isoleucine	8.1 (0.7, 16.1)	8.6 (−4.2, 12.3)	0.644
Valine	22.7 (−9.9, 39.3)	1.5 (−5, 38.6)	0.683
Phenylalanine	**1.2 (−2.8, 5.4)**	**−4.5 (−6.5, −2.6)**	**0.024**
Tyrosine	**3.2 (−4, 9.1)**	**−9.7 (−15.1, −4)**	**0.018**
Lysine	**17.5 (−1.5, 24.3)**	**−11.5 (−31.4, 0.5)**	**0.036**
Arginine	**6.1 (−2.2, 10.1)**	**−21.0 (−31.8, −10.9)**	**0.005**
Methionine	1.7 (−0.4, 3.1)	2.3 (−0.1, 3.1)	0.765
Glutamine	0.7 (−25.0, 44.1)	24.3 (−74.5, 87.1)	0.644
Threonine	2.4 (−11, 13.8)	3.0 (0.1, 21.7)	0.289
Alanine	−2.7 (−35.9, 42.9)	9.5 (−26.9, 35.4)	0.724
Aspartic acid	**0.2 (0.0, 0.4)**	**−0.3 (−0.6, −0.1)**	**0.018**
Glutamic acid	**4.3 (−5, 6.5)**	**−10.1 (−15.9, −5.6)**	**<0.001**
Serine #	**−3.1 (−8.1, 7.1)**	**14.6 (9.5, 22.3)**	**0.002**
Glycine #	**−0.3 (−14.7, 14.3)**	**26.2 (10.6, 42.8)**	**0.013**
Histidine #	0.6 (−2, 6)	6.4 (−0.7, 9.5)	0.265
1-Methylhistidine	0.3 (−2.6, 5.3)	0.0 (−3, 2.3)	0.644
3-Methylhistidine	−0.1 (−0.3, 0.7)	−0.1 (−0.7, 0.2)	0.765
β-Alanine	0.2 (−0.1, 0.3)	−0.1 (−0.3, 0.3)	0.724
α-Aminoadipic acid	**0.0 (−0.1, 0.1)**	**−0.2 (−0.3, −0.1)**	**0.006**
β-Aminoisobutyric-acid #	0.0 (−0.1, 0.1)	0.2 (0, 0.2)	0.061
α-Amino-N-butyric-acid #	0.1 (−2.5, 1.3)	1.7 (−0.6, 6.9)	0.135
Asparagine #	3.5 (−2.5, 9.7)	10.0 (2.1, 13.3)	0.201
Citrulline	**2.9 (−1.3, 3.9)**	**−8.9 (−12.2, −7.1)**	**<0.001**
Ornithine #	2.0 (−3, 7.4)	−0.1 (−4, 6.6)	0.849
Taurine	−1.9 (−6.6, 4.9)	−2.9 (−5.2, 2.1)	0.978
Ethanolamine	**0.4 (−0.1, 0.9)**	**−1.3 (−1.6, −0.9)**	**<0.001**
Sarcosine	0.1 (0.0, 0.3)	0.0 (0.0, 0.1)	0.369
Proline #	10.2 (−13.2, 29.6)	37.7 (8.6, 63)	0.097
Hydroxyproline	0.2 (−0.2, 0.4)	0.6 (−0.5, 2)	0.683
Hydroxylysine 2 #	0.0 (0.0, 0.1)	0.0 (0.0, 0.1)	0.497
Cystine #	2.2 (−11.1, 4.9)	−2.3 (−6.9, 5.3)	>0.999
Tryptophan	−0.9 (−2, 3.4)	−3.9 (−6.8, −1.4)	0.053
Global Test Statistic, sum of rankings across panel of 33 AA/AA metabolites #	**487 (456, 533)**	**356 (323, 387)**	**<0.001**

These AA/AA metabolites were sorted in descending order when computing the rankings for the global test statistics. The remaining AA/AA metabolites were sorted in ascending order when computing the rankings for the global test statistics. A lower mean value for the global test statistic in the Pioglitizone plus Metformin group is interpretable as this group having larger decreases in the panel AA/AA metabolites over the study period (note: the largest decrease in an AA would results in a ranking of 1).

2.3. *Statistical Methods*

All statistical analyses were performed using SAS software (Version 9.3, Cary, NC). Results are presented as medians with interquartile ranges. Spearman rank correlations (ρ) were used to examine univariate associations between AA/AA metabolites and the GIR at baseline. Wilcoxon Rank Sum tests were used to determine significant differences between treatment groups at baseline and between treatment groups with respect to their pre- to post-intervention change scores (Δ). The sample size of insulin sensitizer study (n = 25) was not conducive to principal components analysis, but we wanted to test for systematic changes over the entire panel of AA/AA metabolites between groups. To do this, O'Brien's nonparametric global test statistic was used [35]. This test is an overall (omnibus) test for differences in a composite score based on the ranked order of Δ for each AA/AA metabolite in the panel. Briefly, the Δ observed with treatment for each AA/AA metabolite was ranked either ascending or descending order based on the correlation with the Δ in GIR. The sum of the ranks for each AA/AA metabolite by person was used as the dependent variable for the global test statistic. The SAS MACRO %GlobTest was used for implementation of the global test [36]. Statistical significance was set at $\alpha = 0.05$ for all comparisons.

3. Results

We previously reported the clinical and demographic characteristics of participants [29]. In brief, the participants had a mean age of ~52 y, BMI of ~31 kg/m^2, body fat of ~45%, fasting glucose of ~126 mg/dL, fasting insulin of ~13 μU/mL and steady-state glucose infusion rate during the hyperinsulinemic clamp of ~25 μmol/kgFFM/min [29]. All of these outcomes are consistent with a phenotype of overweight/obesity and T2D or high risk for T2D.

Spearman rank correlations (ρ) revealed inverse correlations between the glucose infusion rate and several AA/AA metabolites at baseline. As expected, the glucose infusion rate was inversely correlated with the BCAA: leucine (ρ = −0.52, p = 0.007), isoleucine (ρ = −0.41, p = 0.043), and valine (ρ = −0.51, p = 0.010); and AAA: phenylalanine (ρ = −0.70, p = 0.007), tyrosine (ρ = −0.55, p = 0.005). In addition, the glucose infusion rate was also inversely correlated with methionine (ρ = −0.56, p = 0.004), tryptophan (ρ = −0.47, p = 0.018), lysine (ρ = −0.41, p = 0.044), and α-aminoadipic acid (ρ = −0.49, p = 0.012).

Table 1 presents the baseline fasting plasma AA/AA metabolite concentrations by treatment group. At baseline, there were no significant differences between treatment groups for any of the fasting plasma AA/AA metabolites concentrations (p > 0.05).

As previously reported [29] three months of insulin sensitizer therapy reduced fasting plasma glucose and insulin concentrations (all p < 0.001). Insulin sensitizer therapy also increased insulin sensitivity estimated the steady-state glucose infusion rate during the hyperinsulinemic-euglycemic clamp (p < 0.001) as previously reported [29]. Compared to placebo, insulin sensitizer therapy significantly also increased QUICKI [11.9% (6.4 −15.6%) vs. −2.4% (−4.2 −0.6%), p < 0.001].

In response to three months of insulin sensitizer therapy plasma BCAA concentrations were not significantly reduced as hypothesized (Table 2). However, the plasma phenylalanine and tyrosine concentrations were reduced (Fig. 1A–B and Table 2) as were the plasma concentrations of glutamic acid, arginine, citrulline, aspartic acid, lysine, α-aminoadipic acid, and ethanolamine (Fig. 1**C**–**I** and Table 2). In contrast, insulin sensitizer therapy increased the plasma serine and glycine concentrations (Table 2). When the O'Brien's global test statistic was applied to the entire AA/AA metabolite panel to determine the overall effect of treatment, there was a highly significant difference between groups (p < 0.001) (Table 2). Moreover, the global rank score was inversely associated with change in glucose infusion rate (ρ = −0.74, p < 0.001).

Spearman rank correlations (ρ) between the absolute pre-to-post intervention change in the glucose infusion rate and the absolute pre-to-post intervention changes in the plasma BCAA and AAA in the insulin sensitizer treated group revealed no statistically significant associations. Likewise, the Spearman rank correlations (ρ) between the relative pre-to-post intervention percent change in the glucose infusion rate and the relative pre-to-post intervention percent changes in the plasma BCAA and AAA in the insulin sensitizer treated group revealed also no statistically significant associations. These data indicate that improvements in insulin sensitivity in response to dual insulin sensitizer therapy can be dissociated from changes in BCAA and AAA.

As described in the reference study on the effects of acute insulin infusion [34], plasma concentrations of leucine, isoleucine, valine, phenylalanine, tyrosine, lysine, arginine, methionine, glutamine, alanine, aspartic acid, glutamic acid, serine, glycine, histidine, and 3-methylhistidine declined (all p < 0.05) in young adults during the 7 hour insulin infusion compared to a control group receiving saline. For the current report we expanded the panel of metabolites measured in that study and found that compared the saline infusion, the insulin infusion reduced plasma concentrations of citrulline [−56% (−45%, −37%) vs. -8% (−11%, −6%)], proline [−37% (−39%, −36%) vs. -15% (−16%, −7%)], ornithine [−41% (−44%, −37%) vs. -4% (−7%, −4%)], hydroxyproline [−30% (−35%, −28%) vs. -8% (−18%, −4%)], α-aminoadipic acid [−34% (−41%, −33%) vs. 2% (−13%, 8%)], taurine [−13% (−23%, −6%) vs. 3% (−2%, 5%)], hydroxlysine-2 [−6% (−11%, −3%) vs. 6% (3%, 9%)], β-aminoisobutyric acid [−49% (−52%, −46%) vs. 21% (7%, 60%)], asparagine [−36% (−43%, −35%) vs. -11% (−15%, −7%)], and α-amino-n-butyric acid [−60% (−62%, −58%) vs. 13% (2%, 27%)] (all p < 0.05).

4. Discussion

The present investigation demonstrates that three months of dual insulin sensitizer therapy (metformin plus pioglitazone) on average reduces fasting plasma AA and AA metabolite concentrations in overweight/obese adults with fasting hyperglycemia or previously untreated T2D. The major new finding was that insulin sensitizer therapy resulted in reduced concentrations of three functional pairs of AA/AA metabolites: 1) phenylalanine and tyrosine, 2) lysine and α-aminoadipic acid, and 3) arginine and citrulline. The same AA/AA metabolites also declined in response to a single 7-hour insulin infusion. Together, these findings support the premise that enhanced insulin action *per se* is responsible for the changes in these functional pairs of AA/AA metabolites, rather than being attributable to a more direct effect of the pharmacological agents, pioglitazone and/or metformin.

An important finding of the present investigation is that dual insulin sensitizer therapy in overweight/obese adults with fasting hyperglycemia or previously untreated T2D reduces plasma concentrations of both phenylalanine and tyrosine (Fig. 1A–B and Table 2). The combination of these two insulin sensitizers is commonly used in T2D. Of interest, increase in insulin sensitivity was not significantly correlated with the decline in fasting plasma phenylalanine or tyrosine. This may indicate that the reduction in these amino acids in response to improvements in insulin sensitivity has a floor effect and not a dose response. A previous report demonstrated that three months of metformin monotherapy reduces fasting AAA in people with T2D [17]. Moreover, three months of pioglitazone has also been shown to reduce fasting AAA concentrations in people with NASH [37].

We hypothesized that insulin sensitizer-induced improvement in insulin sensitivity would translate into reduced fasting concentrations of plasma BCAA. Although insulin sensitivity increased, plasma BCAA concentrations were unchanged (Table 2). Skeletal muscle protein breakdown, adipose tissue degradation of BCAA by BCKD, and subsequent oxidation of branched chain ketoacids (BCKA) by mitochondria play important roles in regulating fasting plasma BCAA concentrations [19,38]. Plasma BCAA concentrations may not have changed due to opposing effects of metformin and pioglitazone. It was shown, for example that two days of metformin therapy increased plasma BCAA in insulin resistant adults [18]. In contrast, pioglitazone, a peroxisome proliferator-activated receptor-γ (PPARγ) agonist, promotes the degradation of BCAA by increasing the activity of BCKD in adipose tissue [39] and has also been shown to reduce fasting

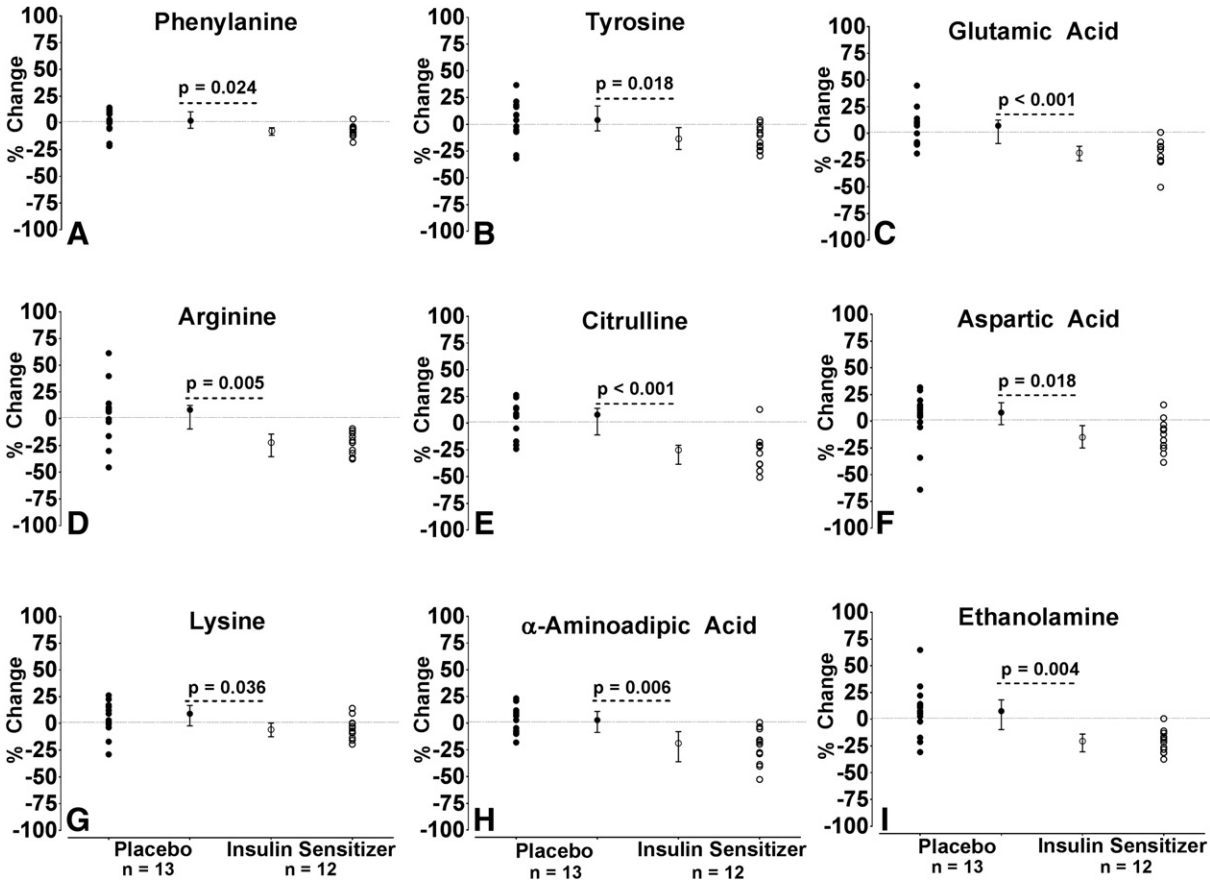

Fig. 1 – Effects of three months of insulin sensitizer therapy on amino acids and amino acid metabolites. Twenty five overweight/obese (BMI ~30 kg/m^2) adults with impaired fasting glucose or untreated diabetes were randomized to three months of pioglitazone (45 mg per day) plus metformin (1000 mg twice per day, N = 12 participants) or placebo (n = 13). Compared to placebo, insulin sensitizer therapy increased fasting plasma concentrations of phenylalanine (A), tyrosine (B), glutamic acid (C), arginine (D), citrulline (E), aspartic acid (F), lysine (G), α-aminoadipic acid (H), and ethanolamine (I). The values represent the individual percent changes for the insulin sensitizer (open circles) and placebo (closed circles) groups. In addition, the median percent change and interquartile ranges are also provided. p-Values are from the Wilcoxon Rank Sum Test for the difference between the absolute change scores using the normal approximation.

plasma BCAA in obese patients with non-alcoholic fatty liver disease [37]. Six months of rosiglitazone, another PPARγ agonist, also reduces BCAA in patients with T2D [40]. It is also feasible that the decline in fasting insulin offsets the increase in insulin sensitivity and insulin-mediated suppression skeletal muscle protein breakdown. In support of this possibility, the insulin sensitizer treatment had no effect on plasma 3-methylhistidine, a byproduct of myofibrillar protein breakdown. The decline in fasting insulin could also reduce the uptake of amino acids into peripheral tissues. Indeed, elevations in insulin secretion associated with sitagliptin therapy lead to reduced circulating BCAA concentrations following a mixed meal in patients with T2D [41]. Our findings are also consistent with a previous report in which it was shown that an alteration in insulin action on glucose metabolism following insulin treatment may not be accompanied by a change in the insulin effect on protein metabolism [42]. Other plausible explanations for the lack of effect of insulin sensitizer therapy on BCAA concentrations include an adaptation of BCAA metabolism to chronically high circulating insulin in people with insulin resistance, or a blunted effect of insulin sensitizers on BCAA transamination, a process that is typically increased in people with diabetes [26,43]. A change in dietary protein intake could also be impact circulating BCAA [44] but participants in the current study consumed a standard diet for three days prior to both the baseline and post-intervention studies to minimize the effect of diet as a confounding variable.

Although some previous studies showed that pharmacological doses of AA, especially BCAA, reduced *in vitro* and *in vivo* insulin action, BCAA in the physiological range appear to play a critical role in maintaining or enhancing insulin sensitivity. For example, dietary leucine supplementation enhanced insulin action in high-fat fed rodents [45,46]. Leucine, and its metabolite β-hydroxy-methylbutyrate (HMB), can act synergistically with pharmacological agents to increase

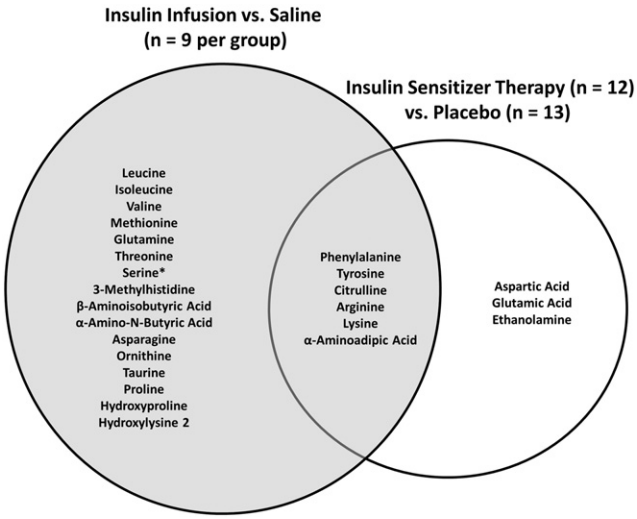

Fig. 2 – Venn diagram for plasma amino acid and amino acid metabolites that were reduced in response to an acute or chronic increase in insulin action. Compared to saline, 22 amino acids and amino acid metabolites were reduced in response to an acute infusion of insulin in healthy young adults (n = 9 per group) (grey circle). Compared to placebo, nine amino acids and amino acid metabolites were reduced in response to three months of insulin sensitizer therapy in overweight/obese (BMI ~30 kg/m^2) adults with impaired fasting glucose or untreated diabetes (n = 13, placebo; n = 12 insulin sensitizer) (white circle). Three functional pairs of amino acids and amino acid metabolites (phenylalanine/tyrosine, lysine/α-aminoadipic acid, and arginine/citrulline) were reduced in response to both the acute infusion of insulin as well as three months of insulin sensitizer therapy. Finally, serine concentrations were reduced in response to the acute infusion of insulin, while it was increased in response to three months of insulin sensitizer therapy.

their potency [47–49]. When combined with metformin, leucine and HMB improved insulin sensitivity by activating AMPK and SIRT1 [47]. Leucine content is high in dairy products and has been identified as a key component for lowering the risk for T2D [50].

In the present investigation insulin sensitizer therapy reduced the concentration of lysine, and its metabolite α-aminoadipic acid (Fig. 1G and H and Table 2). In the presence of hyperglycemia, lysine can be converted to allysine through a non-enzymatic Strecker-type reaction [51]. Oxidative stress and/or low concentrations of the anti-oxidant glutathione lead to further oxidation of allysine to α-aminoadipic acid [52]. As a result, α-aminoadipic acid has been proposed as a potential biomarker of protein oxidation in T2D [53], is elevated in people with diabetes [53], and is a predictor for the future development of T2D [33]. We previously demonstrated that insulin withdrawal for 8 hours increased oxidative stress, which was reflected by an accelerated oxidation of lysine to allysine in *de novo* synthesized apoA1 [54] and a concomitant elevation in α-aminoadipic acid in people with type 1 diabetes [32]. Therefore, we speculate that the insulin sensitizer induced reductions in α-aminoadipic acid reflect reductions in overall oxidative stress mediating further improvements in insulin sensitivity.

Arginine and citrulline concentrations declined in response to insulin sensitizer therapy (Fig. 1D–E and Table 2), but the underlying mechanism(s) are not yet clear. Arginine and citrulline are key components of nitric oxide biosynthesis and the urea. Reductions in citrulline likely reflect insulin sensitizer-induced reductions in urea biosynthesis, secondary to reductions in gluconeogenesis [4,55]. Insulin acutely reduces ornithine level affecting the catabolic aspect of arginine metabolism consistent with the anti-catabolic effect of insulin on AA metabolism.

Insulin sensitizer therapy also reduced the concentration of glutamic acid/glutamate (Fig. 1C and Table 2). Glutamic acid/glutamate is an essential precursor for gluconeogenesis so the elevation in glutamic acid/glutamate may contribute to the development of fasting hyperglycemia in insulin-resistant states. Likewise, the reduced fasting glutamic acid/glutamate following pioglitazone and metformin treatment could contribute to a concomitant reduction in gluconeogenesis. Metformin has previously been shown to reduce gluconeogenesis and hepatic glucose production [56] and this effect may be at least partially mediated via reduction in glutamic acid/glutamate concentration, although gluconeogenesis is highly regulated by the rate-limiting enzyme, phosphoenolpyruvate carboxykinase. There is also recent evidence demonstrating that metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerol phosphate dehydrogenase [57]. Taken together, glutamic acid/glutamate may play a permissive role in the regulation of gluconeogenesis.

We found that nine AA or AA metabolites were reduced in response to three months of insulin sensitizer therapy, whereas in the reference study 22 AA or AA metabolites declined in response to an acute insulin infusion (Fig. 2). The higher number of changes in the reference study may be because the participants were all young, healthy adults with high insulin sensitivity who were studied during 7 hours of insulin infusion without a meal or AA supplement. In contrast, the participants undergoing insulin sensitizer treatment were older, had higher body fat, lower insulin sensitivity and were studied in the overnight fasted state. Nevertheless, six AA and AA metabolites were reduced in both studies, and represent three functional pairs: i) phenylalanine and tyrosine, ii) lysine and α-aminoadipic acid, and iii) arginine and citrulline.

Among the strengths of the present study was that it was placebo controlled and double blinded [29]. Secondly, the insulin sensitizer therapy substantially improved insulin sensitivity in all treated participants. A potential limitation is that insulin sensitizer therapy could have altered the plasma AA and AA metabolite concentrations both directly and indirectly. However, the analysis of samples from the reference insulin infusion study [34] clearly demonstrated that changes in AA metabolites can occur if insulin action is acutely increased. Additionally, since we used both pioglitazone and metformin together their separate effects on the study outcomes have yet to be determined.

In summary, three months of insulin sensitizer therapy with metformin and pioglitazone in a double-blind trial results in a global reduction in plasma AA and AA metabolite

concentrations. Elevations in BCAA, AAA, and AA metabolites have the potential to be used as a biomarker for both diabetes risk and monitoring the effects of strategies designed to lower that risk. Importantly, insulin sensitizer therapy significantly reduced three functional pairs of AA/AA metabolites (phenylalanine/tyrosine, lysine/α-aminoadipic acid, and arginine/citrulline). The long-term clinical impact of those changes has not yet been determined. It remains to be determined whether these metabolites could predict individuals who are or are not benefiting from the insulin sensitizing therapy and therefore more or less likely to develop T2D.

Author Contributions

BAI collected data, interpreted data and wrote the manuscript. REC performed biostatistical analyses, interpreted data and revised manuscript. MS, LT, KRS, RB designed study, collected data, and revised manuscript. AW, HS, HK collected data. KSN designed study, supervised collection and analyses of data, interpreted data, and revised manuscript. All authors provided critical feedback to the manuscript.

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Disclosure Summary

We have no conflicts of interest to disclose.

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